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ORIGINAL ARTICLE

Negative relationships between cellular immune response, Mhc class II heterozygosity and secondary sexual trait in the montane water vole

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Abstract

Heterogeneities in immune responsiveness may affect key epidemiological parameters and the dynamics of pathogens. The roles of immunogenetics in these variations remain poorly explored. We analysed the influence of Major histocompatibility complex (Mhc) genes and epigamic traits on the response to phytohaemagglutinin in males from cyclic populations of the montane water vole (*Arvicola scherman)*. Besides, we tested the relevance of lateral scent glands as honest signals of male quality. Our results did not corroborate neither the hypotheses of genome-wide heterozygosity-fitness correlation nor the Mhc heterozygote advantage. We found a negative relationship between Mhc hetetozygosity and response to phytohaemagglutinin, mediated by a specific Mhc homozygous genotype. Our results therefore support the hypothesis of the Arte-Dqa-05 homozygous genotype being a 'good' Mhc variant in terms of immunogenetic quality. The development of the scent glands seems to be an honest signal for mate choice as it is negatively correlated with helminth load. The 'good gene' hypothesis was not validated as Arte-Dqa-05 homozygous males did not exhibit larger glands. Besides, the negative relationship observed between the size of these glands and the response to phytohaemagglutinin, mainly for Mhc homozygotes, corroborates the immunocompetence handicap hypothesis. The Mhc variants associated with larger glands remain yet to be determined.

Introduction

Since the 1990s, ecological immunology has addressed the physiological and molecular bases of variations in immune responsiveness by placing immunity in the context of ecology and adaptation (Schulenburg et al. 2009). Understanding these disparities across individuals or species has major implications in evolutionary ecology as immunocompetence probably represents one of the main components of fitness (Lochmiller and Deerenberg 2000). These variations are also important for zoonosis epidemiology. Heterogeneity in host susceptibility may strongly influence key epidemiological parameters such as parasite intensity, transmission or virulence (e.g. Dowel 2001).

Proximal factors influencing variation in immune defences are multiple, including physiological or environmental factors. They have been largely investigated (for rodents, see Nelson et al. 2002). Besides, the influence of genetics on the intensity of the immune response still remains scarcely explored in natural populations. Major histocompatibility complex (Mhc) genes are relevant candidates to address this question. They encode for glycoproteins that recognize antigens, bind peptides derived from them and present them to lymphocyte T cells (Klein

1986). Therefore, they participate in the initiation of the antigen-specific immune response by inducing communication among different cellular components of the immune system (Klein 1986). In particular, antigen presentation via Mhc class II molecules plays a key role in initiating and maintaining cell-mediated and humoral immune responses.

Few empirical studies have provided evidence for the role of Mhc gene polymorphism in the control of differences in immune responsiveness (e.g. Makhatadze et al. 1995; Apanius et al. 1997; Zhou and Lamont 2003; Kurtz et al. 2004). In natural populations, the role of Mhc alleles was investigated rather than that of Mhc heterozygosity (but see Kurtz et al. 2004 for laboratory experiments; Makhatadze et al. 1995 for human studies). It was thus worthy to assess the effects of Mhc gene heterozygosity on immunocompetence. From a theoretical point of view, Doherty and Zinkernagel (1975) suggested that Mhc heterozygotes should exhibit the highest immunocompetence (the 'heterozygote advantage' HA hypothesis), in terms of the recognition and elimination of pathogens, because such individuals could present a broader range of pathogen-derived peptides. Then empirical works rather focused on the associations between Mhc heterozygosity and resistance/tolerance to one or multiple parasites than on associations between Mhc heterozygosity and immune responsiveness (e.g. Froeschke and Sommer 2005; de Eyto et al. 2007; Oliver et al. 2009).

Different patterns of relationships between Mhc gene heterozygosity and the intensity of immune response might be observed.

On one hand, positive associations are expected under the models of genome-wide heterozygosity-fitness correlation (HFC) or Mhc heterozygote advantage (HA, Doherty and Zinkernagel 1975). Under the HFC hypothesis, positive associations between heterozygosity estimated at neutral markers and the intensity of immune response should also be observed. Significant positive correlations between estimates of genome-wide heterozygosity and cell-mediated immune responses have been found in bird species and corroborate this hypothesis (Reid et al. 2003; Hawley et al. 2005; Hale and Briskie 2007). Several mechanisms, including the decline of host immunity with inbreeding, may underlie HFC patterns (O'Brien and Evermann 1988; Coltman et al. 1999; Keller and Waller 2002; Altizer et al. 2003). Conversely, under the HA hypothesis, no expectation can be made on the association between heterozygosity estimated at neutral markers and the intensity of immune response.

On the other hand, associations might be observed between specific Mhc alleles and the intensity of immune response (e.g. Makhatadze et al. 1995; Bonneaud et al. 2005). Such relationships can be expected under 'good-genes' models of female mate choice (Trivers 1972; Mays and Hill 2004; Neff and Pitcher 2005). Males bearing 'good' alleles, i.e. alleles that increase individual fitness (Andersson 1994) by conferring higher immunocompetence for example, should be preferred. Benefits of such mating are multiple and include direct advantages, i.e. the avoidance of parasitized individuals, as well as indirect ones, i.e. the transmission of these 'good alleles' to offsprings (Hamilton and Zuk 1982). Under this scenario, we might expect females to seek mates carrying specific Mhc alleles, locally adapted against prevalent pathogens or associated with higher immune responses. Assuming that mates homozygous for such 'good' alleles would be even more favoured (e.g. in Salmo salar, Langefors et al. 2001), we would expect a negative relationship between Mhc gene heterozygosity and the intensity of immune response. This relationship would hence only be driven by those particular 'good' Mhc alleles.

Next, studying the relationships between the development of a secondary sexual character and immune responses might provide complementary insights into the mechanisms driving these associations between Mhc gene and immune response.

On one hand, a negative relationship between a secondary sexual trait and immune responses would support the immunocompetence handicap hypothesis (ICHH; Folstad and Karter 1992; Wedekind and Folstad 1994). The ICHH postulates that only males carrying genetic characteristics associated with superior immunocompetence/better disease resistance might afford to allocate more resources to costly ornament traits at the expense of the immune function (Hamilton and Zuk 1982; Folstad and Karter 1992; Wedekind and Folstad 1994). This hypothesis relies on Zahavi's handicap theory, which proposes that individuals that express male epigamic traits are handicapped by a reduced immune response (Zahavi 1975). In vertebrates, this handicap is linked to testosterone, the primary male sex hormone, which is required for the production of many morphological sexual characters, and has a suppressive effect on the immune system (Zuk 1996).

On the other hand, a positive relationship between an honest secondary sexual trait and immune responses would support the 'good genes' hypothesis. Those males carrying 'good genes', especially for parasite resistance, would theoretically be able to afford to invest both in immunity and in secondary sexual traits.

The montane water vole *Arvicola scherman* (*Rodentia, Cricetidae, Arvicolinae*) is an interesting organism for investigating these hypotheses (HFC, HA, Good genes and ICHH). This rodent exhibits regular 5- to 8-year dynamic cycles (Saucy 1994). It is considered as a pest in Western Europe because outbreaks are associated with extensive damages for agriculture. It is also a reservoir for three important (re)-emerging viral zoonoses in Europe, caused

by hantaviruses, orthopoxviruses and arenaviruses (Charbonnel et al. 2008b) and for three agents of priority zoonoses (*Echinococcus multilocularis*, *Leptospirosa* sp., *Toxoplasma gondi*, refs in Giraudoux et al. 2008). Six geographically close localities were sampled during 3 years corresponding to the outbreak and decline phases of *A. scherman* abundance cycles. Previous population genetic studies have shown that phases of increasing abundance and outbreak during *A. scherman* cycles were associated with increase in effective size and migration between populations (Bryja et al. 2007). Consequently, these phases were characterized by low spatial and temporal genetic differentiation.

We used the Mhc genotypes characterized at two class II genes (Dqa and Drb) and published in Tollenaere et al. (2008). We estimated immune responsiveness as the magnitude of cell-mediated immune response using a challenge with phytohaemagglutinin. We measured the flank gland, which is considered as a secondary sexual character of voles (Quay 1968; Jannett 1986). Several arguments support the presumption of flank glands being a male honest sexual signalling in montane water voles: these glands regress after castration and develop in response to exogenous testosterone (Stoddart 1972), they go through an annual activity cycle where the maximal output coincides with the breeding season (Stoddart 1972), during which the dominant males defend their territories against intruders. Finally, the chemical composition of these glands varies between social statuses (Stoddart et al. 1975). The glands produce a characteristic odour (Frank 1956). The results of field and laboratory experiments showed that females preferred the odour of dominant males to that of subordinates ones (Evsikov et al. 1994) and that high-ranking males gained reproductive advantage (Evsikov et al. 1997).

Following the reasoning detailed above, we assessed the first prediction of an association between male response to phytohaemagglutinin and Mhc gene heterozygosity, either positive (HFC, HA hypotheses) or negative ('good genes' hypothesis and association between Mhc alleles and response to phytohaemagglutinin). Next, we tested the pertinence of scent glands as honest signals of male quality in terms of parasite load. We then investigated the associations between the development of these glands and response to phytohaemagglutinin to analyse the relative influence of the immunocompetence handicap and 'good genes' hypotheses.

Materials and methods

Species and sampling

The montane water vole is a small rodent (~80 g) that inhabits subterraneous burrows in open meadows and farmland habitats (Le Louarn et al. 2003). It is subject to pronounced and regular multiannual fluctuations in population abundance in some parts of its distribution, including the Jura Mountains (France and Switzerland). In these areas, *A. scherman* displays abundance cycles over 5- to 8-year periods i.e. 15–25 generations (Saucy 1994; Weber et al. 2002). Our study site Nozeroy (47.11°N, 6.24°E, Franche-Comté, France) is located in these mountains. The area is about 70 km^2 and consists of permanent pastures interrupted by hedges and small forests. Six localities (1–2 ha) were sampled three times in October 2003, October 2004 and October 2005 (Fig. 1). The weak spatial and temporal genetic differentiation previously observed between these sites and years of sampling indicated that genetic structure would hardly influence overall results (Tollenaere et al. 2008). In consequence, individual data were pooled over space and time in further analyses. We used 80 BTS (Besançon Technologie Services) live-traps per locality. In each locality, the traps were set during a single day of capture at a minimum spacing of 5 m (i.e. in different vole colonies, as confirmed by genetic studies based on microsatellite loci Berthier et al. 2006; Tollenaere et al. 2008) to avoid the capture of closely related individuals (parent–young or full siblings). Previous population genetic studies based on microsatellites did not reveal any heterozygote deficit in these samples, suggesting the absence of kin groups (Tollenaere et al. 2008). This capture protocol led to the capture of approximately 20 animals per site and date. On arrival at the laboratory facilities, voles were housed individually in polypropylene boxes with wood shavings and hay bedding. Apples and water were provided *ad libitum* and the animal room was maintained on a 14L:10D cycle with ambient temperature (22°C). Animals were acclimated to captivity for 1 week before assessing immune response. The localities were also surveyed for their relative abundance in montane water voles using surface index method (Giraudoux et al. 1995). Spatio-temporal variations in vole abundances have been described in a previous study for these localities (Charbonnel et al. 2008a).

Response to phytohaemagglutinin

The cell-mediated immune function was assessed using the phytohaemagglutinin delayed hypersensitivity response (*PHA*). This measure provides an estimation of the proliferative response of the circulating T lymphocytes to the injected mitogen (Goto et al. 1978). This particular immune response is very useful for assessing the overall competence of cell-mediated components of immunity within a host and provides a reliable indicator of *in vivo* cellular immunity in rodents (Mendenhall et al. 1989; Sinclair and Lochmiller 2000; Webb et al. 2003; Gouy de Bellocq et al. 2006). The strength of the response to

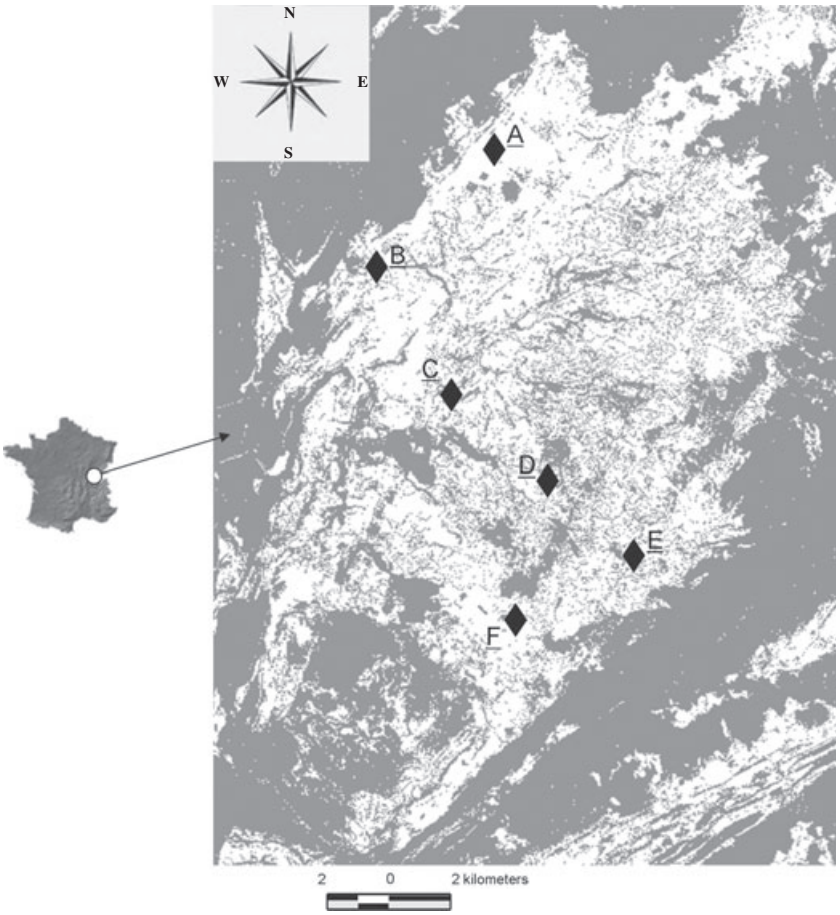

Figure 1 Location of the sampling sites. On the left, situation of the Jura Mountains (Franche-Comté) in France. On the right, map of the Nozeroy canton with the six sampling sites indicated by letters. A = Cuvier, B = Onglières, C = Doye, D = Billecul, E = Fraroz, F = Arsure. Grey and white colours respectively represent favourable and unfavourable habitats of *Arvicola scherman*.

phytohaemagglutinin also seems related to the capacity of hosts to face parasitism (e.g. Christe et al. 2000). Besides, the genetic control of lymphocyte responsiveness to stimulation by phytohaemagglutinin has been identified in laboratory rat (Newlin and Gasser 1973) and mouse (Stiffel et al. 1977) strains. Similarly human and poultry studies have also provided evidence that Mhc genes participate in this genetic control of the phytohaemagglutinin responsiveness (e.g. Batory et al. 1983; Taylor et al. 1986, 1997; Makhatadze et al. 1995). It seems likely that associations/interactions of some Mhc molecules on the cell surface create unique conformational structures, which may in turn affect T-lymphocyte proliferation through the impairment of cellular adhesion and the contact inhibition of growing cells (refs in Makhatadze et al. 1995).

Each animal was injected intradermally in the centre of the right foot with 0.1 mg of phytohaemagglutinin (Sigma product no. L8754) in 30 μL of physiological phosphate-buffer saline. The thickness of the region (inflammation) was measured three times with a pressure-sensitive micrometer (Mitutoyo 547–301) to the nearest 0.01 mm before and 24 h (±15 min) after the injection. Average measures were used in further analyses. The swelling was estimated as the change in thickness of the right foot from the day of injection with phytohaemagglutinin until the following day (minus the change in thickness of the left foot during the same period for the control). One person carried out all injections and thickness measurements. Part of these data has previously been published (Charbonnel et al. 2008a).

Voles were euthanized by cervical dislocation. They were sexed, measured and weighed to the nearest 0.1 g. Only males were considered in this study, therefore reducing by half the total sampling. We estimated the age of individuals using eye lens mass and the formula obtained by Boujard (1982) from a population of *A. scherman*, geographically close to our study area. We also expressed body condition as body mass index (*BMI*; body weight/length, following Schulte-Hostedde et al. 2001).

Sexual maturity, development of a secondary sexual character and parasite load

Montane water voles only exhibit little sexual dimorphism. The most obvious secondary sexual characteristics

are paired lateral scent glands, situated on the flanks on each side of the body (Frank 1956; Quay 1968; Stoddart 1972; Jannett 1986). They are more developed in males than females, and in older than in juvenile males, suggesting either genetic or androgenic dependence (Quay 1968). We measured the biggest length and width of the right gland to the nearest 0.5 mm, and we approximated the surface (*Sgland*) occupied by this pad as the product of these two measures. For a few voles, the hip gland was so small that it could not be observed. In this case, we considered that the size of the hip gland was 0.

Helminths from the liver, the body cavity and the digestive tracts were identified under the microscope, as previously described for some of these samples in Tollenaere et al. (2008). Parasite richness (R_h) was expressed as the number of helminth species detected per vole.

Mhc gene and genome-wide heterozygosity

We used the Mhc and microsatellite dataset published by Tollenaere et al. (2008). First, we verified the absence of inbreeding or related individuals in our samples using these microsatellite data. Deviations from Hardy–Weinberg proportions were quantified for each site and year by the unbiased Wright inbreeding coefficient F_{IS}, estimated as described by Weir and Cockerham (1984) using Genepop 4.0 (Rousset 2008). To confirm the absence of related individuals within samples, we also investigated relatedness coefficients for all pairs of individuals using SPAGeDI v1.2 (Hardy and Vekesman 2002). Relationship coefficients between individuals were estimated over all microsatellite loci (see below) as the proportion of genes in one individual with alleles identical to those of a reference individual. We used the estimation and sample size correction defined by Wang (2002). We performed a Jackknife procedure to provide a standard error of the multilocus estimates.

Mhc genotyping was performed using capillary electrophoresis-single strand conformation polymorphism (CE-SSCP) analysis (Bryja et al. 2006). cDNA cloning and sequencing had previously been performed to identify all potential variants. We noted that Dqa gene in *A. scherman* corresponds to one locus (locus Dqa1: alleles 2,3,5,6,7), which is always amplified, and another locus (locus Dqa2: alleles 1 and 4, not distinguishable by SSCP), which presents a polymorphism of duplication (Bryja et al. 2006). Indeed, some individuals exhibited none of these Dqa2 alleles. Dqa sequencing based on RNA has confirmed that all Dqa1 and Dqa2 alleles are transcribed (Bryja et al. 2006). Genetic diversity was thus described for each vole using five parameters: heterozygosity at loci Dqa1 and Drb (*Dqa1-het*, *Drb-het*), number of amino acid differences between allele sequences for both Dqa1 and Drb loci (genetic distance *Dqa1-dist*, *Drb-dist,* zero for homozygotes), and the presence/absence of the locus Dqa2 (*Dqa2-pres*). Indeed such variation in the number of present loci also contributes to the variability in the number of Mhc molecules. We did not consider Mhc haplotypes as previous population genetic studies of the montane water vole have shown only weak linkage disequilibrium between these genes (Bryja et al. 2007; Tollenaere et al. 2008).

The genome-wide heterozygosity was assessed from nine unlinked autosomal dinucleotide microsatellite loci, developed specifically for *A. scherman* (Berthier et al. 2004). We estimated the standardized individual multilocus heterozygosity (*sMLH*), which is the proportion of heterozygous loci per individual corrected for nonscored loci (see Slate et al. 2004). We also estimated $\bar{d^2}$, the squared differences in repeat units between two alleles in an individual at a given locus averaged over all loci (Coulson et al. 1998).

Statistics

We were interested in how response to phytohaemagglutinin (*PHA*) was affected by Mhc or genome-wide genetic diversity and sexual secondary character development (*Sgld*), while controlling for age (*Age*), body condition (*BMI*), time (*Year*) and space (*Site*). Seven explanatory variables were used to describe Mhc or genome-wide genetic diversity, of which four were continuous (*Dqa1-dist, Drb-dist, sMLH,* $\bar{d^2}$) and three were nominal (*Dqa1-het, Drb-het*, *Dqa2-pres*). Two-way interactions were included in the models.

We first explored the data graphically to ascertain their distribution, to identify outliers and to examine the interdependence between variables. The *PHA* variable was square root-transformed to normalize the distribution of its error structure. A multiple regression analysis was performed with two-way interactions included in the models. We used the Akaike Information Criterion (AIC) to select the most parsimonious model, the one explaining most of the variance with the fewest parameters (Burnham and Anderson 1998; Johnson and Omland 2004). Models with δAIC <2 compared to the model with the lowest AIC were selected. Significance of explanatory variables and their interactions was determined by using deletion testing, with the significance of a term determined by the log-likelihood ratio test (McCullagh and Nelder 1989). When an interaction term was found to be significant, the lower-order terms involved in that interaction were also retained (Crawley 1993).

Models were first validated by the examination of the histogram and of the normal probability plots of residuals to ensure that residuals were normally distributed.

Second, we verified that standardized residuals were fairly evenly distributed when plotted against the fitted values. When outliers were identified in residual plots, the models were refitted with these observations removed to determine their effects on parameter estimation.

We expected high correlations among individual variables. This collinearity is known to hamper model selection in regression analyses (Graham 2003). In particular, it makes parameter estimates unstable and inflates standard errors (Quinn and Keough 2002). Using sequencial Bonferroni correction, collinearity was identified between *Dqa1-dist* and *Dqa1-het*, *Drb-dist* and *Drb-het*, *Drb-dist* and *Dqa2-pres*, *Drb-het* and *Dqa1-het*, *Sgland* and year, *BMI* and both *Sgland* and year. The potential confounding effects of these explanatory variables were considered during model selection. The models that included collinear variables were refitted with each variable removed to determine misleading variations in coefficients. Finally, when a significant relationship was observed between response to phytohaemagglutinin and Mhc heterozygosity, we examined the specific associations between Mhc genotypes or alleles and immune response intensity using analysis of variance (ANOVA) and sequential Bonferroni corrections. Multiple comparison tests were assessed using Tukey–Kramer HSD (honestly significant difference) test (Tukey 1953). These last analyses were only performed for the Dqa1 locus (5 Dqa1 alleles and 15 Dqa1 genotypes) as the Drb locus was so polymorph (13 Drb alleles and 46 Drb genotypes) that analyses would have suffered from very low statistical power.

In parallel, we tested differences in development of scent glands between males exhibiting different parasite load using an ANOVA. Five males had helminth richness larger than 4 and were grouped in a single class.

All analyses were performed using GENSTAT 7.1 (Lawes Agricultural Trust, Rothamstead).

Results

This study relies on 150 males sampled between 2003 and 2005 at six localities. Because of low abundance, no voles could be sampled at localities A, B and C in 2005.

Analyses on within sample genetic structure confirmed the absence of kin groups or related individuals that could have biased our results. F_{IS} estimates were low and not significant, as well as relatedness coefficients (Table 1).

An extreme value was identified (*PHA* = 1.4 cm, next highest values are *PHA* = 1.28 cm, *PHA* = 1.26, *PHA* = 1.22). We decided to remove it as it was highly nonrepresentative (10% more than the next values) and could strongly bias the modelling process. We nevertheless checked that including this point would not have modified our conclusions but only the probabilities associated with our results. After the selection procedure, the final model was defined as follow: *PHA* ~ *Drb-dist* + *Dqa1-het* + *Sgld* + *Dqa1-het.Sgld* + *Age* + *Year* (Table 2). Genome wide heterozygosity had no effect on *PHA*. A clear Mhc effect was observed, with voles homozygous at Dqa1 having higher levels of response to phytohaemagglutinin than heterozygotes ($P = 0.046$, Fig. 2). This result was also found for the Drb locus, but in a lesser extent: heterozygotes with highly distant allele sequences had lower response to phytohaemagglutinin than homozygotes or heterozygotes with more similar allele sequences ($P = 0.044$). A negative association was found between the response to phytohaemagglutinin and the surface of the flank gland. A tendency was observed for the whole dataset, and this was significant when considering Dqa1 homozygotes, as indicated by the significant interaction observed between the surface of the flank gland and the Dqa1 heterozygosity ($P = 0.039$, Fig. 3). The response to phytohaemagglutinin was also positively correlated with age ($P < 10^{-4}$) and a temporal effect was detected where responses to phytohaemagglutinin were higher in 2005 than in 2004, in 2004 than in 2003 and in 2005 than in 2003 (all $P < 10^{-4}$).

The influence of collinearity between *Year* and *Sgland* was assessed by refitting the model without the covariable *Year*. This did not affect the directionality of the coefficient for *Sgland*. However, we observed that removing the

Table 1. Within population genetic characteristics estimated over all microsatellites for the different sites and years of sampling. F_{IS} estimates and the associated exact test probability of Hardy–Weinberg equilibrium HW (*P*) are provided. The relatedness coefficients are estimated following Wang (2002) and the standard error (SE) is computed from a jackknife procedure over all loci.

Year and site of sampling	Microsatellite F_{IS}	HW (*P*)	Relatedness coefficient, mean (SE)
2003			
A	0.065	0.871	−0.0158 (0.0164)
B	0.018	0.930	0.0217 (0.0297)
C	−0.002	0.509	0.0196 (0.0361)
D	0.004	0.463	0.019 (0.031)
E	−0.030	0.187	0.0132 (0.0304)
F	0.043	0.958	0.0219 (0.0323)
2004			
A	0.019	0.951	−0.0138 (0.0125)
B	0.064	0.975	−0.0195 (0.0128)
C7	0.057	0.659	−0.0137 (0.0185)
D5	0.028	0.786	−0.0139 (0.0128)
E	0.038	0.801	0.0101 (0.0159)
F	−0.025	0.398	−0.0091 (0.0140)
2005			
D	−0.018	0.518	0.0143 (0.0173)
E	−0.080	0.015	0.0012 (0.0133)
F	0.016	0.779	0.0245 (0.0149)

Table 2. Summary of the retained terms and coefficients (standard errors and probabilities) of the selected models (AIC = 145.83, % variance = 42.7, $F_{12,130} = 9.83$, $P < 10^{-4}$).

Terms	Comparisons	Coefficients (SE)	*t*	*P*-value
Dqa1-het	Homozygote vs. heterozygote	−0.0881 (0.0418)	−1.94	0.03
Drb-dist		−0.0043 (0.0023)	−2.04	0.04
Sgland		−0.0006 (0.0005)	−1.05	0.31
Dqa1-het.Sgland		0.0016 (0.0007)	2.08	0.03
Age		0.0094 (0.0026)	3.26	$<10^{-4}$
Year	2003 vs. 2004	0.1741 (0.0257)	6.75	$<10^{-4}$
	2003 vs. 2005	0.2088 (0.0302)	7.11	$<10^{-4}$
	2004 vs. 2005	0.0348 (0.0276)	2.12	$<10^{-4}$

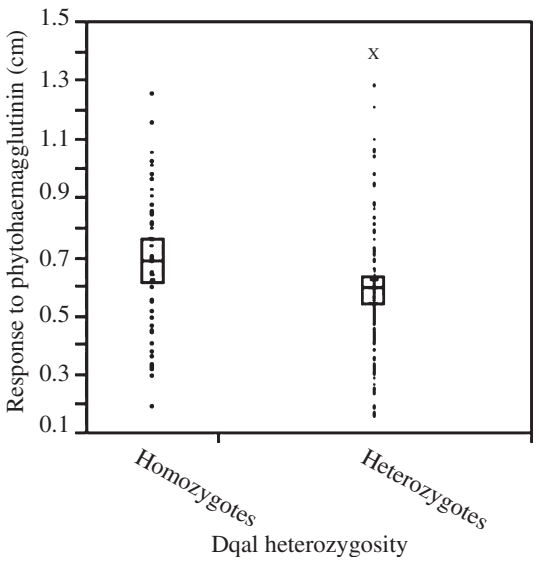

Figure 2 Relationship between response to phytohaemagglutinin and Dqa1 heterozygosity (*Dqa1-het*). The quantile box-plot indicates the mean and its 95% confidence interval. The cross symbol refers to the individual that was considered as an outlier and removed from the analyses.

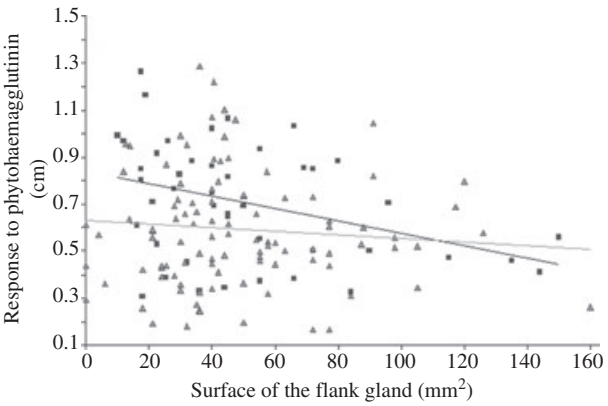

Figure 3 Relationship between response to phytohaemagglutinin and the surface of the flank gland for Dqa1 homozygotes (black squares and black line) and Dqa1 heterozygotes (grey triangles and grey line).

covariable *Year* made the variable *Sgland* become significant ($P = 0.032$) and decreased the significance level of the *P*-value coefficient associated with the interaction *Dqa1-het.Sgland* ($P = 0.063$). We thus decided to retain both variables *Year* and *Sgland* in the selected model.

No significant relationships were observed between response to phytohaemagglutinin and Dqa1 alleles ($P > 0.05/5$ for all tests). Dqa1 genotypes significantly explained response to phytohaemagglutinin (ANOVA, $F_{14,134} = 2.374$, $P = 0.005$). Using the Tukey–Kramer HSD test, we showed that this result was mediated by the Arte-Dqa-05/Arte-Dqa-05 genotype, which exhibited significantly higher levels of response to phytohaemagglutinin than other Dqa1 genotypes (see Fig. 4).

Helminth data were available for 131 voles only. Four nematodes (*Trichuris arvicolae, Syphacia nigeriana, Aonchotheca* sp. and *Eucoleus bacillus*) and four adult cestodes (*Anoplocephaloides dentata, Paranoplocephala gracilis, Paranoplocephala omphalodes and Arostrilepis horrida*) have been identified. Helminth richness ranged between 0 and 5. A significant relationship was observed between the surface of the flank gland *Sgland* and the specific richness in helminth ($F_{4,126} = 3.091$, $P = 0.018$). The gland of unparasitized males was more developed than the gland of males harbouring more helminths.

Discussion

This study constitutes one among the rare works investigating the relationships between Mhc gene heterozygosity and the intensity of an immune response in natural populations, considering both the effects of neutral and selective evolutionary processes (see for seminatural populations, Eizaguirre et al. 2009).

No evidence of heterosis or inbreeding depression acting on response to phytohaemagglutinin

No significant association was identified between genome-wide heterozygosity estimated at neutral microsatellites

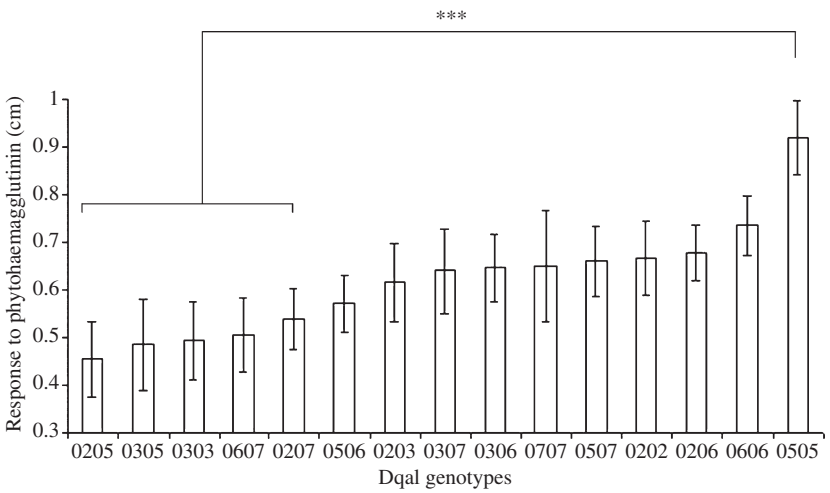

Figure 4 Relationship between response to phytohaemagglutinin and Dqa1 genotypes. Error bars represent ±1SE of the mean. Stars indicate the five Arte-Dqa genotypes exhibiting significantly lower levels of *PHA* than the homozygous Arte-Dqa-05 genotype using *post-hoc* Tukey–Kramer tests.

and the response to phytohaemagglutinin. This result allowed withdrawing the hypothesis of a genome-wide heterozygosity-fitness correlation based on this estimate of immunity. It suggested that there was no inbreeding depression acting on the response to phytohaemagglutinin (Slate et al. 2004). It also showed that none of these microsatellite markers exhibited overdominance or was in linkage disequilibrium with other overdominant markers (Hansson et al. 2004). Besides, this absence of genome-wide heterozygosity-fitness correlation suggested that the strong bottlenecks experienced by *A. scherman* populations through regular multi-annual abundance cycles did not result in inbreeding acting on vole response to phytohaemagglutinin. This assumption was also supported by the absence of heterozygote deficiencies observed at microsatellites in these samples (see also Bryja et al. 2007; Tollenaere et al. 2008). This was most likely explained by important effective population size and gene flow quickly recovered after abundance declines (Berthier et al. 2006; Bryja et al. 2007).

The advantage of Arte-Dqa-05 homozygous genotype in response to phytohaemagglutinin

We demonstrated that homozygote males at Dqa1 and Drb Mhc genes exhibited significantly higher responses to phytohaemagglutinin than heterozygote ones. We also revealed that this positive relationship between the response to phytohaemagglutinin and Mhc homozygosity was mostly mediated by a particular Dqa1 genotype, involving the Arte-Dqa-05 allele. The polymorphism observed at the Drb gene was unfortunately too high to allow discriminating the alleles or genotypes mediating the relationship between Drb heterozygosity and response to phytohaemagglutinin. Implications of Mhc alleles in the response to phytohaemagglutinin have previously been observed in birds and humans (Makhatadze et al. 1995; Bonneaud et al. 2005). They may be explained by interactions of Mhc molecules on the cell surface that could influence the impairment of lymphocyte proliferation (Makhatadze et al. 1995).

Mate choice for immune 'good alleles' might arise if particular Mhc variants confer strong benefits against the pathogenic environment (Hamilton and Zuk 1982). We can assume that the higher response to phytohaemagglutinin developed by Arte-Dqa-05 homozygote voles could indicate a lower parasite load for these individuals. Our previous analyses of the genetic/parasitological associations occurring within these montane water vole populations provide arguments in favour of this scenario. Tollenaere et al. (2008) have shown that the Arte-Dqa-05 allele was negatively associated with the richness in gastro-intestinal helminths, although this association could not be confirmed by cross-validation (when dividing the dataset in two independent ones, we found that this association was significant in only one of the dataset, probably because of a lack of statistical power). Female voles that would mate with Arte-Dqa-05 homozygous males in these populations would gain the direct benefit of mating with less parasitized males and the indirect benefit of transmitting 'good' immune alleles to their offsprings. A similar example of mate choice driven by immune 'good alleles' has been described in sticklebacks (Eizaguirre et al. 2009). Males that exhibited the highest probability to be chosen by females carried a specific Mhc haplotype, which was associated with higher resistance against a common and virulent parasite.

Besides, Arte-Dqa-05 homozygote male voles tended to exhibit less developed lateral glands than other voles (results not shown). This result suggested that these males

carrying 'good alleles' with regard to immunity were not able to afford a strong investment in this potential sexually selected trait. We can thus suggest that the positive correlation between the intensity of the response to phytohaemagglutinin and particular Mhc genotypes was more explained by the immunocompetence handicap hypothesis than by the 'good gene' hypothesis, what we confirmed below.

Glands as honest signals of male genetic quality and immunocompetence handicap

We found that the development of the lateral scent glands of male voles was negatively correlated with helminth load. Moreover, several studies from the literature supported the hypothesis that mate choice of female water voles was based on the development and odour of male flank glands. Wolff and Sherman (2007) reported female mate preferences and differences in male reproductive success in *A. terrestris*. Experiments demonstrated that females preferred the odour of dominant males to that of subordinate ones (Evsikov et al. 1994; cited in Wolff and Sherman 2007). These odours could originate from the flank gland. Indeed it was shown in meadow voles that scents from the male posterolateral region contained sexual/social preference information, and that the attractiveness of these scents was completely dependent on gonadal hormones (Ferkin et al. 1994). A last argument of the literature corroborating these scent glands as an honest signal of male genetic quality in rodents was that male scent marking rates and gland sizes were heritable traits (e.g. in bank voles Horne and Ylönen 1998). It is thus likely that scent glands are secondary sexual traits that function as indicators of resistance to parasites, which might be important for parasite-mediated sexual selection (Hamilton and Zuk 1982) or intra-sexual competition (e.g. Fox and Hudson 2001).

We also found a negative association between the response to phytohaemagglutinin and the development of the flank gland. This result is in agreement with Zahavi's handicap (Zahavi 1975). The development of the flank gland could lead to immunosuppression, indirectly through increased nutritional cost (Lochmiller 1996; Sheldon and Verhulst 1996) or directly as androgenic hormones required for its development have immunosuppressive effects (Folstad and Karter 1992). Two arguments could support the hypothesis of a trade-off mediated by testosterone between response to phytohaemagglutinin and secondary sexual character in *A. scherman*. First, there is no obvious evidence for a direct physiological link between immunocompetence and flank gland development. Second, Stoddart (1972) showed that the flank gland of male montane water voles regressed after castration and developed in response to exogenous testosterone. It is thus probable that testosterone is simultaneously immunosuppressive and involved in the development of the flank gland.

This handicap was mostly observed when considering Mhc homozygous males. Physiological data are missing to elucidate these differences between Mhc homozygotes and heterozygotes. The fact that blood testosterone levels and responses to testosterone injections seemed to be genetically associated with the Mhc system in mice, when studying Mhc congenic lineages (Ivanyi 1978), could explain these differences. More recently, chemical analyses of Mhc-congenic male mice have even shown that Mhc genes influenced the amounts of testosterone-mediated pheromones contained in urinary odours (Novotny et al. 2007). Therefore, it could now be interesting to complete these experiments by investigating the relationships between testosterone and Mhc heterozygosity.

Under the immunocompetence handicap hypothesis, we expected that only males carrying 'good' Mhc variants in terms of resistance to parasitism would afford to allocate more resources to the development of lateral glands at the expense of the immune function. We previously showed that male voles carrying the homozygous Arte-Dqa-05 genotype, identified as 'good alleles' in terms of resistance to disease, also exhibited higher levels of response to phytohaemagglutin as well as smaller glands (result not shown). This Mhc variant might thus not underlie the immunocompetence handicap. The Mhc alleles or genotypes responsible for the good immune genetic quality of males exhibiting large glands and low response to phytohaemagglutinin remain to be determined. Other studies have shown that secondary sexual traits, known to influence mate choice, were correlated to Mhc genes (e.g. Buchholz et al. 2004; Jager et al. 2007).

Our results have highlighted a higher response to phytohaemagglutinin associated with a particular Mhc homozygous genotype, and a trade off between this response and the development of a secondary sexual character during *A. scherman* outbreak and decline. Interesting perspectives could concern the investigation of these patterns between immunity, immunogenetics and epigamic traits in the course of *A. scherman* abundance cycles. Trade offs might be exacerbated during outbreak and decline, because of substantial nutritional stress associated with high abundance. They might therefore not occur during phases of low abundance. In addition, parasitism pressure as well as most common or virulent pathogens might also change in intensity and direction throughout *A. scherman* abundance cycle. 'Good' Mhc variants associated with better disease resistance could thus differ among cycles.

This would reinforce balancing selection maintaining Mhc polymorphism in *A. scherman* vole cyclic populations.

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